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Title: Parasite-mediated host behavioural modifications: *Gyrodactylus turnbulli* infected
Trinidadian guppies increase contact rates with uninfected conspecifics

Running title: Adaptive guppy responses to *Gyrodactylus* infection

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SUMMARY

While group formation provides antipredatory defences, increases foraging efficiency and mating opportunities, it can be counterintuitive by promoting disease transmission amongst social hosts. Upon introduction of a pathogen, uninfected individuals often modify their social preferences to reduce infection risk. Infected hosts also exhibit behavioural changes, for example, removing themselves from a group to prevent an epidemic. Conversely, here we show how Trinidadian guppies infected with a directly transmitted ectoparasite, *Gyrodactylus turnbulli*, significantly increase their contact rates with uninfected conspecifics. As uninfected fish never perform this behaviour, this is suggestive of a parasite-mediated behavioural response of infected hosts, presumably to offload their parasites. In the early stages of infection, however, such behavioural modifications are ineffective in alleviating parasite burdens. Additionally, we show that fish exposed to *G. turnbulli* infections for a second time, spent less time associating than those exposed to parasites for the first time. We speculate that individuals build and retain an infection cue repertoire, enabling them to rapidly recognise and avoid infectious conspecifics. This study highlights the importance of considering host behavioural modifications when investigating disease transmission dynamics.

Key words: *Gyrodactylus*; *Poecilia reticulata*; sociality; infectious disease; transmission dynamics; behavioural modification

KEY FINDINGS

- Gyrodactylus turnbulli infected guppies significantly increase contact rates with conspecifics.
- This adaptive behavioural response of the host is presumably aimed at ‘offloading’ parasites.
- In the early stages of infection, such behaviour is not an effective strategy for reducing parasite burdens.
- Fish exposed to secondary infections associated for less time than those experiencing primary infections.
- This study highlights the importance of considering adaptive host behavioural changes when investigating disease dynamics.

INTRODUCTION

Sociality confers many benefits, particularly with respect to antipredatory defences (Krause and Ruxton, 2002; Sansom *et al.* 2009). Group formation can, however, be counterintuitive by increasing competition for scarce resources, mating opportunities, and the conspicuousness of prey to predators, all of which can directly impede fitness (Alexander, 1974; Sherman *et al.* 1995). Most notable is increased disease susceptibility owing to chronic stress induced by social competition (Glaser and Kiecolt-Glaser, 2005; Proudfoot and Habing, 2015; Schneider *et al.* 2016), and the close proximity of conspecifics facilitating disease propagation amongst hosts (Côté and Poulin, 1995). This often results in more intense, prevalent infections as group size increases (reviewed in Patterson and Ruckstuhl, 2013).

At a population level, a significant determinant of a disease transmission pathway is a group's social structure, which arises from consistent interaction patterns between conspecifics (Whitehead, 1997; Wey *et al.* 2008). The rate of disease transmission depends on the 'socialness' and infection status of group members. Well-connected individuals, for example, are at greater risk of acquiring infections (Bell *et al.* 1999; Danon *et al.* 2011), and can subsequently act as superspreaders of disease (Lloyd-Smith *et al.* 2005; Craft, 2015). As infection becomes more prevalent within a population, conspecifics often respond to visual and/or chemical cues indicative of infection by modifying their associative preferences to reduce their infection risk. Such behavioural avoidance has been documented in a number of taxa including amphibians (Kiesecker *et al.* 1999; Koprivnikar and Penalva, 2015), fish (Ward *et al.* 2005) and mammals (Curtis, 2014; Poirotte *et al.* 2017).

Infected hosts often show remarkable adaptive behavioural traits aimed at combatting disease. Infected fish, for example, frequent warmer thermal conditions outside the thermal tolerance of their associated pathogens to 'self-medicate' against infection (see Mohammed *et al.* 2016). Alternatively, infected hosts can isolate themselves to protect the integrity of a group. Extreme forms of altruistic behaviour, such as 'altruistic suicide', have been observed within social insects including ants and honeybees; upon acquiring infection, these hosts remove themselves from a colony to prevent disease epidemics (Heinze and Walter, 2010; Rueppell *et al.* 2010). Conversely, by forming larger groups, parasite 'attacks' can become diluted amongst group members (Duncan and Vigne, 1979; Rätti *et al.* 2006), with such encounter-dilution effects mitigating parasite-mediated costs of sociality (Mooring and Hart, 1992).

Whether or not infected individuals increase their contact rates with uninfected conspecifics remains questionable. Indeed, ‘offloading’ parasites onto conspecifics could be beneficial in terms of alleviating parasite burdens and their associative costs, whilst also serving to ‘vaccinate’ recipients against subsequent infections (Faria *et al.* 2010). Using the well-studied social Trinidadian guppy and its directly transmitted ectoparasite, *Gyrodactylus turnbulli*, we investigate how parasitism drives adaptive behavioural responses in hosts, which could aid in alleviating parasite burdens. Specifically, we investigate (a) if association times and direct contact patterns between fish change depending on infection status, (b) assess the significance of these behavioural responses in determining successful parasite transmission, and (c) assess how the parasite infrapopulation influences transmission dynamics, irrespective of behaviour.

MATERIALS AND METHODS

Ethics statement

All applicable institutional and/or national guidelines for the care and use of animals were followed. Procedures and protocols were conducted under UK Home Office license (PPL 302876) with approval by the Cardiff University Animal Ethics Committee.

Host and parasite origin

Experimental Trinidadian guppies (*Poecilia reticulata*) were laboratory-reared descendants of a wild stock caught from the Lower Aripo River, 2012. Fish were initially housed at Exeter University, before being transferred to Cardiff University in October 2014. Here, fish

were maintained in 70L dechlorinated water tanks under standard conditions of $24 \pm 1^\circ\text{C}$ on a 12 h light: 12 h dark photoperiod (lights on 07:00-19:00), and fed daily with Aquarian tropical fish flakes subsidised with *Daphnia magna* and *Artemia* spp.

Experimental fish were infected with *Gyrodactylus turnbulli* (strain Gt3), originating from, and subsequently maintained on, an inbred ornamental guppy stock since 1997. A single parasite was isolated onto a naïve ‘culture’ fish, and following successful establishment reproduced forming a highly inbred parasite population. This population has since been maintained under standard conditions in culture pots, each containing a minimum of four naïve culture fish collectively infected with *ca.* 30 *G. turnbulli* worms. Naïve fry were added to the culture pots biweekly to prevent parasite extinction.

Experimental design

Only female guppies were used during experimental trials due to their increased propensity to shoal compared to males (Griffiths and Magurran, 1998). In the wild, females typically form small shoals (2-20 individuals), between which males move in search of mating opportunities (Croft *et al.* 2004). Thus, by excluding males we eliminated potentially confounding factors associated with mating attempts in influencing parasite transmission. Unfamiliarised female guppies were size-matched according to their standard length (SL; $\pm 4.1\text{mm}$) into pairs ($n = 50$), and individually housed in 1L-dechlorinated water under standard conditions. As infection histories of these fish differed, we categorised dyads into five treatments summarised in Table 1. Treatment 1 utilised parasite naïve guppies and formed a control group. During trials these fish were sham infected to account for handling time but never exposed to parasites. Naïve guppies were also used for Treatment 2, which were

experimentally infected with a predetermined dose of *Gyrodactylus turnbulli* worms ('Primary infection': see Table 1), and tested 24 h post infection. Treatments 3, 4 and 5 comprised dyads that had experienced *G. turnbulli* infection in a previous experiment (ranging from 20-40 worms), but had been clear of parasites for a minimum of three months prior to this experiment. During a trial, one fish in each dyad was experimentally infected with 40 *G. turnbulli* worms ('Secondary infection'), and tested 24 (Treatment 3), 48 (Treatment 4) and 72 h (Treatment 5) post-infection.

Behavioural trials took place in a partitioned glass tank (30 x 30 x 45cm) filled with 7L-dechlorinated water and maintained at $24 \pm 1^{\circ}\text{C}$. A removable translucent barrier separated dyads prior to a trial, allowing visual but not physical contact. The tank was placed in an experimental chamber surrounded by white fabric on three sides to prevent external disturbance, with one side left open for behavioural observations. A 2cm² grid attached to the sides of the tank enabled visual estimation of the distances between conspecifics. The arena was lit from above using daylight mimicking strip lights (Sylvania T5 F13W/54-765 G5 Luxline Standard Daylight bulb) diffused by white fabric.

Experimental procedure

Each dyad underwent a two-stage trial comprising behavioural and parasite transmission procedures. On Day 1, dyads were placed into the partitioned experimental tank for a 24 h acclimation period. On Day 2, both guppies were transferred from the tank to individual 1L-dechlorinated water pots using a plastic container. One fish in each dyad was then infected with a predetermined number of *Gyrodactylus turnbulli* worms (see Table 1 for *G. turnbulli* dose and sample sizes). Experimental infections entailed sacrificing a heavily infected culture

fish, and placing it in close proximity to a temporarily anaesthetised recipient fish (using 0.02% buffered MS222), allowing direct transmission of worms. The infection process was observed continuously under a dissection microscope with fibre optic illumination, and once infected a recipient was revived in 1L-dechlorinated water. It's uninfected conspecific was sham infected to control for handling time and each pair returned to the partitioned tank for a subsequent 24 h, thus ensuring fish were familiar with this environment to minimise exploratory behaviour during a subsequent trial.

On Day 3, both fish were removed from the partitioned tank, as above, temporarily anaesthetised and screened under a dissection microscope to quantify the starting *G. turnbulli* intensity of a donor, and confirm the uninfected status of their conspecific. The number of individual worms was counted three times to ensure exact intensities were recorded. Variability in *G. turnbulli* start intensity, particularly for treatments 4 and 5 (see Table 1) were observed given the parasites 24 h reproductive potential (Bakke *et al.* 2007). Fish were returned to the partitioned tank for 10 min prior to removal of the translucent barrier, which allowed dyads access to one another. A 10 min behavioural trial began when one guppy crossed the central partition line. During a trial, the proportion of time dyads spent associating was quantified (seconds) using a stopwatch based on direct observation. An association was defined as when fish were \leq four body lengths from one other; a standard measure of shoaling (Pitcher, 1983). Additionally, the number of direct contacts initiated by the infected fish was tallied. Infected guppies exhibit clamped fins (Cable *et al.* 2002), and appear to 'rub-up' against conspecifics, although previously only anecdotal evidence of such behaviour exists (Croft *et al.* 2011; Stephenson *et al.* 2017). This unique behavioural response of infected fish coupled with pathological symptoms of infection made the identity of each fish obvious during a trial. A direct contact was therefore defined as when an infected fish

instigated skin-skin contact (typically lasting <1 sec) with an uninfected conspecific, which is particularly important for facilitating *G. turnbulli* transmission (Bakke et al. 2007). Following a behavioural trial, both fish were again individually removed from the partitioned tank, temporarily anaesthetised and screened to quantify successful transmission of individual *G. turnbulli* worms

To assess the significance of *G. turnbulli* infrapopulation intensity on potential *G. turnbulli* transmission dynamics, guppies remained anaesthetised following screening, and were placed in the same petri dish submerged in 1 cm depth dechlorinated water. Using a pipette tip, fish were manipulated so that they were in direct contact with one another. The number of *G. turnbulli* worms to transmit from a donor to a recipient fish was then monitored for a 5 min period. At the end of a trial, guppies were chemically treated using 0.1% levamisole and screened clear of infection, on 3 consecutive inspections, before returning to stock tanks.

Statistical analysis

Statistical analyses were performed in R (3.0.2; R Core Team 2013). Using the *lme4* library (Bates et al. 2014), two Generalised Linear Mixed Effects Models (GLMMs), fitted with binomial family and ‘logit’ error structures, were used to assess variables influencing the proportion of time dyads spent associating (the dependent term in both models). The first model included donor *Gyrodactylus turnbulli* intensity, dyad mean SL, treatment, and interactions between each variable, as independent terms. Treatment 1 data was excluded from this model, as there was no possible relationship between the uninfected control dyads and parasite intensity. To address differences in dyad association time between treatments (including Treatment 1), a second GLMM was performed whereby dyad mean SL, treatment

and an interaction between these terms were included in a model as independent terms, with dyad association time the dependent term. Parasite intensity was excluded from this model. Both models included dyad ID as a random term to account for repeated measures.

Using the *glmmADMB* package (Bolker *et al.* 2008) a negative binomial GLMM was used to investigate the effects of dyad mean SL, donor *G. turnbulli* intensity, association time and treatment on the total number of direct contacts observed between dyads, instigated by an infected donor. Interactions between association time x *G. turnbulli* intensity and treatment x *G. turnbulli* intensity were incorporated into the model. A final negative binomial GLMM investigated the significance behavioural trials, transmission trials, dyad mean SL, treatment and donor *G. turnbulli* intensity had on determining total *G. turnbulli* transmission. Dyad ID was incorporated into each model as a random term to account for repeated measures. Models were refined via the sequential removal of non-significant terms to minimise Akaike Information Criterion (Pinheiro and Bates, 2000; Bates *et al.* 2014), and model robustness assessed using residual plots.

RESULTS

Behavioural trials

Infection with *Gyrodactylus turnbulli* significantly influenced the proportion of time dyads spent associating (GLMM: $z = -7.27$, $SE = 0.022$, $P < 0.001$), which also differed between experimental treatments (GLMM: $z = -4.33$, $SE = 0.189$, $P < 0.001$). For fish infected with *G. turnbulli* up to 24 h (Treatments 2 & 3), the time dyads spent associating increased with parasite intensity (Fig. 1a). Conversely, for dyads experiencing secondary infections of 48

and 72 h, association time decreased as a donor infection intensified (Treatments 4 & 5, respectively: Fig. 1a). Dyad mean SL significantly influenced association time, which also depended on treatment (Dyad mean SL x Treatment interaction, GLMM: $z = -10.48$, SE = 0.004, $P < 0.001$). For control dyads and naïve fish enduring a 24 h primary *G. turnbulli* infection (Treatment 2), larger dyads spent more time associating (Fig. 1b). For fish infected with 24, 48 and 72 h secondary infections (Treatments 3, 4 and 5, respectively), the relationship between dyad shoaling time and mean SL was less pronounced (Fig. 1b).

Association time and *G. turnbulli* intensity significantly influenced the number of direct contacts initiated by an infected donor towards its uninfected conspecific. Specifically, more direct contacts occurred the longer dyads spent associating (GLMM: $z = 1.98$, SE = 0.767, $P < 0.005$), and as a donors parasite intensity increased (GLMM: $z = 2.80$, SE = 0.029, $P = 0.0309$; Fig 2a). However, only on two occasions did successful *G. turnbulli* transmission occur as a consequence of host behaviour.

Gyrodactylus turnbulli transmission

When investigating the potential for *Gyrodactylus turnbulli* transmission following host behavioural trials (i.e. placing an anaesthetized donor fish in direct contact with an uninfected recipient for a 5 min period), we found that the total number of *G. turnbulli* to transmit was significantly influenced by a donors' parasite intensity (GLMM: $z = 2.09$, SE = 0.006, $P = 0.037$). As parasite intensity increased, the number of parasites transmitting to a recipient also increased (Fig. 2b). The duration of infection did not affect total *G. turnbulli* transmission (Treatment main effect: GLMM: $z = 1.08$, SE = 0.10, $P = 0.278$).

DISCUSSION

Here we show that infected fish significantly increase direct contact rates with conspecifics. As uninfected fish did not perform such behaviour, we speculate that this parasite-driven host behavioural response attempts to reduce parasite burdens. Additionally, we show that association times between fish exposed to secondary *G. turnbulli* infection was less than those experiencing infection for the first time. We speculate that fish build an infection cue repertoire, enabling them to rapidly identify and subsequently avoid infectious conspecifics.

Our results are consistent with Croft *et al.* (2011), who provided anecdotal evidence of infected guppies initiating direct body contact with conspecifics. Although gyrodactylid infections do cause host behavioural modifications (see Bakke *et al.* 2007), these are merely by-products of infection (e.g. Kolluru *et al.* 2009). Behavioural manipulation by *G. turnbulli* would imply host neurochemical interference (Adamo and Webster, 2013), which has previously never been shown by these ectoparasites. Furthermore, *G. turnbulli* worms did not exhibit a strong transmission propensity during behavioural trials, indicating that increased contact rates are likely an adaptive behavioural response of the host as opposed to parasite manipulation.

Alleviating ectoparasite burdens often involves hosts participating in interspecific, intraspecific and/or self-grooming behaviours (reviewed in Hart, 2011). Intraspecific grooming is particularly evident in higher animal taxa, most notably mammals such as ungulates. Reciprocal allogrooming in impala (*Aepyceros melampus*), for example, significantly reduced tick infestations by up to 95% that of controls (Mooring *et al.* 1996). Fish also engage in grooming activity, whereby cleaners including gobies, wrasse or shrimps

remove clientele ectoparasites, which provides nutritional and health benefits for both parties, respectively (Grutter, 1996; Whiteman and Côté, 2002; Titus *et al.* 2017). Cleaning services are typically concentrated to tropical coral reefs (Côté, 2000), although freshwater intraspecific examples do exist (e.g. Common carp; Soto *et al.* 1994). For fishes that do not engage in cleaning services, alternative parasite removal strategies are employed. For example, physical dislodgement of parasites through skin abrasion against substrata (Urawa, 1992), behavioural fever (Reynolds *et al.* 1976; Mohammed *et al.* 2016), or as observed here increasing contact frequencies with uninfected conspecifics in the early stages of infection. The number of direct contacts instigated by infected guppies towards their uninfected conspecifics significantly increased with *G. turnbulli* intensity. Direct contact between hosts governs *G. turnbulli* transmission dynamics (see Johnson *et al.* 2011), and it could be expected that parasite transmission increase linearly with direct contact frequencies. Contrary to our predictions, successful *G. turnbulli* transmission only occurred twice during behavioural trials, irrespective of the number of direct contacts between infected and uninfected fish. This indicates that such behavioural responses adopted by a host were ineffective in reducing parasite burdens, at least in the initial stages of infection.

Transmission dynamics are mediated by a complexity of factors from both host and parasite perspectives (see Stephenson *et al.* 2017). Ectoparasites adopting a direct transmission strategy risk dislodgment and subsequent mortality. Dislodged *G. turnbulli*, for example, have a maximal off host survival of 31 h at 25°C (Schelkle *et al.* 2013). In the initial stages of infection when the parasite infrapopulation is low, worms do not compete for host resources and therefore the transmission risk outweighs the cost of remaining on an initial host. Although not examined here, in the later stages of infection competition for resources due to a greater parasite density, coupled with activation of the host's immune response (Buchmann

and Bresciani, 1998), may prompt parasite transmission away from unfavourable host microhabitats (Boeger *et al.* 2005; Pie *et al.* 2006).

For parasite naïve controls, dyad association time increased with mean SL. Similarly, this trend was observed for dyads experiencing a 24 h primary *G. turnbulli* infection, although a slight decrease in association time was evident. Larger fish generally have greater shoaling tendencies than smaller, younger individuals (Pitcher *et al.* 1983; Paxton, 1996; Rodgers *et al.* 2011). This may be associated with ‘safety in numbers’ as large individuals are more conspicuous to predators. Fish experiencing secondary infections, however, only associated when infection intensities were low. These individuals may have developed a chemical cue repertoire of infection, which overrides their social preferences irrespective of size. During development, individuals imprint on both behavioural and chemical cues of conspecifics to build a ‘phenotypic template’, which is important for social decisions later on in life (see Mateo, 2004). Using these templates, individuals decipher between normal and abnormal cues emitted from conspecifics, to which they can respond accordingly. Such cues important in communicating the infection status of individuals could be associated with a host’s immune response.

Fish exhibit both innate and acquired immune responses to gyrodactylid infections (e.g. Scott and Robinson, 1984; Scott, 1985; Cable and van Oosterhout, 2007), which are directed to the hosts’ epidermis (Richards and Chubb, 1996). Here, immune by-products including host complement (Buchmann, 1998), changes in mucosal secretion composition (Moore *et al.* 1994), and cortisol release into the surrounding water (Stoltze and Buchmann, 2001) translate into chemical cues indicative of infection. Additionally, fish may also perceive excretory compounds of ectoparasites, although to date this remains unknown. Detection of these cues

subsequently mediates avoidance behaviours to reduce infection risk, particularly in the late stages of infection (Stephenson and Reynolds 2016). Furthermore, the combination of both innate and acquired immunity during secondary infections could have emitted stronger 'infection cues' resulting in a significant reduction in the association times between infected and uninfected fish exposed to 48 and 72 h secondary *G. turnbulli* infections. It should be noted, however, that acquired resistance diminishes post-recovery (Scott, 1985; Cable and van Oosterhout, 2007), and as our experimental fish had previously been uninfected for over 3 months, it is unlikely that acquired immune responses of infected fish contributed to the production of cues indicative of infection. Alternatively, the development and retention of a chemical cue repertoire of infection during primary parasite exposure may have been important in instigating evasive fish behaviours during subsequent infection exposure. Such parasite-mediated avoidance behaviour is particularly beneficial considering the severe pathological costs of gyrodactylid infection (reviewed in Bakke *et al.* 2007).

To conclude, this study shows that in the early stages of infection, parasite-driven behavioural modifications of infected hosts is not an effective strategy for reducing parasite burdens. Such behavioural responses may have greater significance in determining parasite transmission during the later stages of infection, when infra-parasite competition and the host's immune response are more pronounced. However, as infection progresses the benefits may be short lived, particularly if the parasite's reproductive rate outweighs a host's offloading ability. Successful transmission as a consequence of increased contact rates would also be costly to conspecifics, and is counterintuitive to the evolutionary theory of sociality. Although we provide the first quantified example of such behaviour, it may be more widespread within the Animal Kingdom as infected hosts attempt to alleviate their infections. Finally, this study provides evidence that fish exposed to secondary parasite infections may

have developed infection repertoires, enabling them to instigate evasive behaviours towards infectious conspecifics sooner than fish experiencing infection for the first time.

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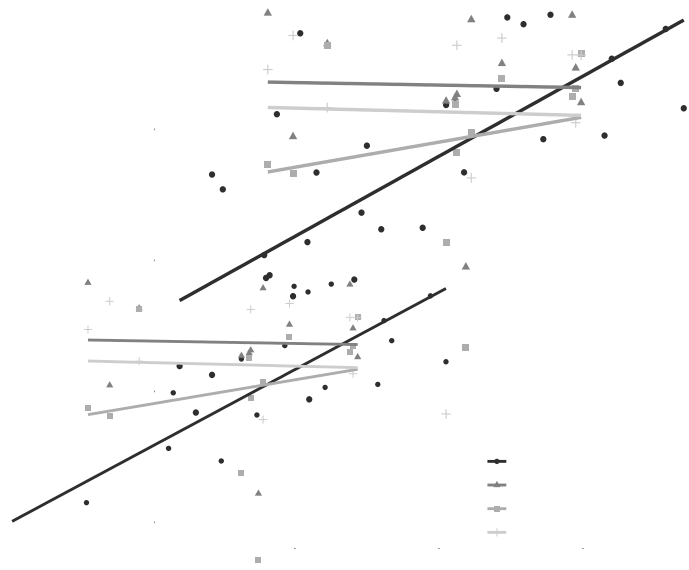
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TABLE 1. A summary of the experimental treatments (1-5) including *Gyrodactylus turnbulli* exposure status (primary or secondary infection), dyad sample size (n), *G. turnbulli* dose administered and infection time before a behavioural trial.

Treatment	Primary or secondary <i>G. turnbulli</i> infection	Dyad sample size (n)	<i>G. turnbulli</i> intensity dose	Infection period (h)
1 (control)	NA	17	NA	NA
2	Primary	15	10 worms	24
		10	20 worms	
		6	40 worms	
3	Secondary	12	All 40 worms	24
4	Secondary	12	Range: 41-72 worms	48
5	Secondary	10	Range: 36-90 worms	72



596

597 **FIGURE 1.** The relationship between the proportion of time dyads spent shoaling and (a)
 598 *Gyrodactylus turnbulli* intensity of the donor fish, and (b) dyad mean standard length (mm).
 599 The solid lines represent the regression between the proportion of time dyads spent
 600 associating on (a) donor *G. turnbulli* intensity, and (b) dyad mean standard length.

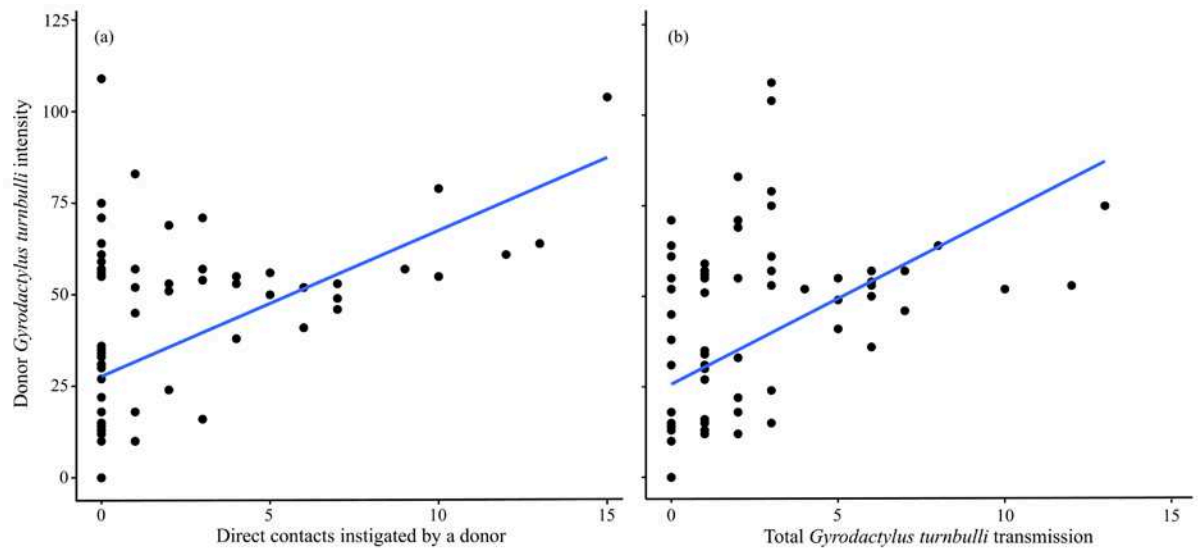


FIGURE 2. Positive association between donor *Gyrodactylus turnbulli* intensity (a) the number of direct contacts instigated by an infected donor fish, and (b) the number of parasites to transmit to an uninfected recipient over a 5-minute duration when both fish were anaesthetized and placed in direct contact. The solid line represents the regression between a donor's *G. turnbulli* intensity on (a) the number of direct contacts instigated by a donor, and (b) the number of worms transmitted to an anaesthetized recipient host.